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PHD THESIS

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BUDAPEST
2012.

1. Introduction

In the Carpatian Basin the thickness of the lithosphere is 24-26 km, it is thinner in 10 kms than that of the other areas of Europe. It largely contributes to the fact that also the value of the thermal step rising from the core of the Earth so geothermal gradient is higher (5°C/100 m) compared to the world-average (approx. 3°C/100 m). The geothermal gradient has different values within Hungary as well: it is larger on the Great Plain (Alföld) and Transdanubia and smaller on highlands. In our country the springs have a temperature above 30°C can be considered as thermal waters (thermal springs). Thermal water can be developed from different aquifers layering on or wedging in each other on the 70-80% part of the country area. So our rich thermal water stock – arising from the thin lithosphere and the good heat insulating capacity of the covering formations – is owing to the advantageous geothermal gradient.

A number of our lukewarm (18-30°C) and thermal water springs have curative water quality, so they have healing power due to their chemical composition. These waters of healing power in Hungary are suitable primarily for calming locomotors and skin diseases, gynaecological complaints and cardiovascular troubles and for improving the operation of the digestive system as a drinking cure. Such waters having healing power are the lukewarm and thermal waters which can be found in Harkány. The advantageous effects on locomotor diseases can be brought into connection with the reduced sulphur compounds of these waters.

The significant thermal ground-water resources of Hungary made the domestic spa and wellness tourisms an important branch. In our country balneotherapy as a form of the communal recreation, has age-old traditions. The composition of our thermal and medicinal waters of different healing powers is various because of the chemical and microbiological processes moving in the depths.

2. Aims of the study

One of our objectives was to examine the characteristic morphologic types of the bacteria and the components of abioseston with phase contrast and electron microscopes in the water of karstic well waters serving Harkány Spa.

Another objective of the present work was to explore the composition and phylogenetic diversity of the bacterial communities, especially the sulphur bacteria participating in forming the chemical composition of the curative water.

It was set as an additional objective after the spring, summer and autumn seasonal sampling and knowing the geological and flow conditions to survey the occurring seasonal and temporal changes in the structures of the bacterial communities.

Another objective was to do a comparative analysis on the explored bacterial communities by using statistic methods.

3. Materials and Methods

In order to achieve the set aims, water samples were collected from four wells of the Harkány Spa (Büdöstapolca-I, Büdöstapolca-II, Matty, Thermal VI) between 2006 and 2008 (Table 1.).

Table 1. Sampling times at the sampling sites and marking of the examinations performed with the taken samples

	Büdöstapolca I well		**Büdöstapolca II well**		**Matty well**		**Termál-VI well**	
	DGGE	Cloning	DGGE	Cloning	DGGE	Cloning	DGGE	Cloning
2006. 11.	-	X	-	X	-	-	-	-
2007. 04.	X	X	X	X	-	-	X	-
2007. 06.	X	-	X	-	X	-	X	-
2007. 08.	X	-	X	-	-	-	X	-
2008. 04.	X	-	X	-	X	X	X	-
2008. 08.	X	-	X	-	X	-	X	-
2008. 11.	X	-	X	-	X	-	X	X

The formed structure of the biomasses given from the water samples were studied by phase contrast microscope and a scanning electron microscope.

Detailed revelation of the structure of bacterial communities was implemented using the molecular cloning method based on the examination of 16S rRNA gene. The composition of the bacterial communities, characteristic to each well was defined by nucleotide sequence analysis of molecular clones. The seasonal and temporal changes of the phylogenetic diversity of bacterial communities were followed by means a molecular fingerprint method, the denaturing gradient gel electrophoresis (DGGE). The changes in the communities were determined by comparing the DGGE patterns and on the basis of the identification of the 16S rDNA sequences obtained from the DGGE bands.

For determining the differences between the clone libraries, a *Bray-Curtis* similarity index was calculated and for estimating the species richness a rarefaction curve was set up.

4. Results and Discussion

During the phase contrast microscopic examinations in the biomass of samples from the lukewarm wells *Thiothrix*-like filaments and trichomes were found. In the samples, intracellular sulphur deposits of different refraction and light transmission could be observed. During recording sulphur with scanning electron microscope iron-sulphur granules, so called framboids were detected as abiotic components of the biomass in creation of which both mineralisation processes taking place in anoxic-reductive thermal medium as well as metabolic processes of the sulphate reducing and iron reducing bacteria could act.

DNA-sequences from 72 group representative molecular clone were identified which represented 46 different phylotypes. The clones had the closest phylogenetic relationship to chemolithotrophic sulphur bacteria, Epsilonproteobacteria clones, sulphate reducing bacteria and other anaerobic sulphur reducing bacteria, as well as different heterotrophic bacteria. Furthermore, two additional (Gram-positive) taxa were detected by analysing the sequences obtained during the DGGE examinations.

The composite sample (BTK) taken from the Büdöstapolca I and II wells in November 2006 consisted of 76 clone sequences which represented 9 different phylotypes. This was the most numerous clone library the major part of which community was constituted by the representatives of sulphide- and sulphur oxidizing chemolithotrophic bacteria of the classes Gamma- and Epsilonproteobacteria, as well as clones related to sulphate reducing Deltaproteobacteria. Additionally, other sequences related to sulphur reducing Deltaproteobacteria and related to chemolithotrophic Betaproteobacteria were identified in a smaller ratio. From among the five clone libraries the BTK was the one which consisted of exclusively sequences related to sulphur bacteria belonging to the phylum Proteobacteria. Within the *Bacteria* domain the Proteobacteria is one of the newest phyla in the phylogeny including the most known genera and species.

The 46 clones originating from the BT1 clone library represented 10 phylotypes which were the members of the phylum Proteobacteria excepting one clone. The Epsilonproteobacteria clones represented in the largest part (35 clones) had the closest relations with anaerobic sulphur oxidizing chemolithotrophic species. Moreover, in the BT1 community different representatives of microaerophil, sulphur oxidizing chemolithotrophic Betaproteobacteria could be found. However, no sequences related to chemolithotrophic Gammaproteobacteria and sulphate reducing bacteria could be shown in this clone library.

Only one clone in relation to an anaerobic sulphur reducing species could be identified which could act in the sulphur reducing processes.

For processing the BT2 clone library only 14 representative clone could be identified during the nucleotide sequence analysis, they represented 10 different phylotypes and belonged to the Proteobacteria with one exception. Because of the low number of clones in the BT2 clone library no real dominance relations could be determined; however the taxonomic composition of the clone library was largely similar to the composition of the BTK library. The representatives of Epsilonproteobacteria were in the closest relation to the same anaerobic chemolithotrophic sulphur oxidizing bacteria as the Epsilonproteobacteria clones found in the BTK and BT1 communities. The clones related to the sulphate reducing Deltaproteobacteria revealed in the BT2 library showed sewuence similarities with the "SRB" clones found in the BTK library. Furthermore, also the BT2 library contained sequences affiliated with BTK clones related to chemolithotrophic sulphide and sulphur oxidizing bacteria belonging to the Beta-and Gammaproteobacteria.

From the hydrodynamic point of view the karstic water system of Matty is of mixed genetics and it was reflected in the composition of the bacterial community, since the clone library of Matty was only partly similar to the clone libraries of Büdöstapolca. The representatives of chemolithotrophic Beta-, Gamma- and Epsilonproteobacteria which were typical for the bacterial communities of Büdöstapolca appeared also in the clone library of Matty, at the same time representatives of Deltaproteobacteria and sulphate reducing bacteria and other sulphur reducing bacteria were missing. Nevertheless, contrary to the clone libraries, until now representatives of a number of heterotrophic soil bacteria were identified here, as well. The members of the clone library consisting of 18 clones were distributed uniformly between 13 different phylogenetic groups so no real dominance relations could be determined as well.

During the comparison of the physico-chemical profiles of water samples taken from the four wells, the largest difference was shown by the water of the thermal well, especially in the water temperature and the sulphide concentration. This difference appeared also in the composition of the clone libraries because the representatives of Epsilonproteobacteria and sulphate reducing bacteria, as well as the filamentous *Thiothrix* species did not appeared in the T6 library. The totally largest difference was found between the clone library of Matty and that of T6; only one common taxon (*Sulfuritalea*) could be found in both libraries. This environment is a good model of the ancient, thermal, reductive, anoxic habitats. The ancient character was shown not only in the physico-chemical parameters of the water of the thermal

well but partially in the composition of the bacterial community as well. During the analysis of the T6 clone library 48 clones representing 9 phylotypes were identified. The most abundant community-forming group (34 clones) was represented by a Betaproteobacteria clone forming a clade with an anaerobic chemolithotrophic species (*Sulfuritalea*) which could be found both in the lukewarm samples form Büdöstapolca and those of Matty. Additionally, in the sulphide and sulphur oxidizing processes ancient anaerobic chemolithotrophic (Aquificae) and anaerobic phototrophic bacteria (Chlorobi) branching far from the Proteobacteria participated and it could be shown from the T6 clone library.

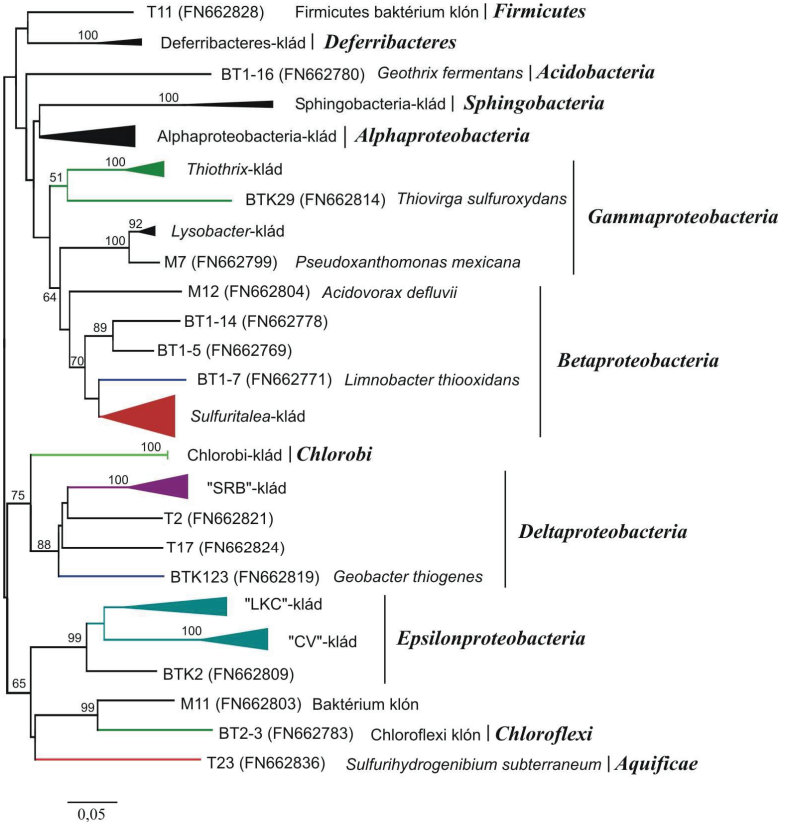

Figure 1. Phylogenetic tree made with the Neighbor-joining method showing the clades and genealogical branches formed by the molecular clones from the examined wells of Harkány Spa
(Bar: 5 % nucleotide difference. Only bootstrap values above 50% are shown (1000 replications)).

Those community forming clones which appeared generally in the five clone libraries but had different diversities per sampling place were environmental Epsilonproteobacteria clones, sulphate reducing bacteria related and chemolithotrophic sulphur bacteria related clones participating in the sulphur cycle. The bacterial community members were arranged into clades according to their genealogical connections and the phylogenetic connections between the clades were represented on a phylogenetic tree (Figure 1.).

From the results of the DGGE analysis the conclusion can be drawn that the seasonal dynamics originated from the weather changes were less and the spatial difference resulted from the geological distance was more expressed (Figure 2.).

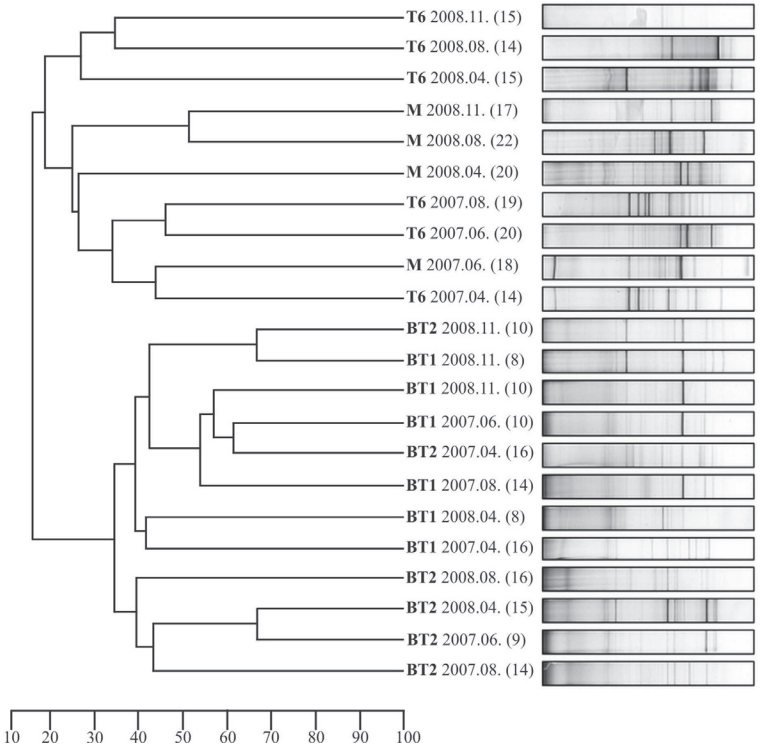

Figure 2. UPGMA dendrogram made on the basis of the DGGE band patterns of bacteria communities originated from the water of examined wells

(Piece number of DGGE bands is in parentheses. Bar: similarity value of the band patterns in percents. BT1: sample taken from the Büdöstapolca I well, BT2: sample taken from the Büdöstapolca II well, M: sample taken from the Matty well, T6: sample taken from the Thermal VI well)

The similarities and differences between the bacterial community structures obtained from the Büdöstapolca I, Büdöstapolca II, Matty and Thermal VI wells reflected primarily the physico-chemical main profile differences of waters as well as the different hydrodynamic relations and mixing of the waters of various genetics of different extent.

According to the *Bray-Curtis* similarity indices the smallest difference could be shown between the libraries BTK and BT1. The values in the middle range were given by the comparison of the clone libraries BTK/BT2, BTK/M, BT1/BT2, BT2/M, as well as BT2/T6. It is interesting that the second lowest value was obtained from the comparison of the communities BT2/M. This relatively considerable similarity was not proven by the results of either the clone library analysis or DGGE similarity dendrogram. It is assumed that the anomaly calculated with the *Bray-Curtis* model was caused by community deforming effect of the small size of BT2 and M clone libraries. The highest *BC* index values were shown by the T6 and M clone libraries so the community structures of these clone libraries differed from each other to the highest degree. The *BC* indices belonging to the T6 library were usually higher than the values belonging to the other clone libraries. The conclusion can be drawn that the community structure of the clone library from the water of Thermal VI well differed also from the other communities to the highest degree. In the background of the high-extent difference of the T6 library is supposedly the physico-chemical profile of the water of Thermal VI well significantly differing from the water of the other producing wells to which the microbial community differing from the communities from lukewarm wells could adapt.

Each clone community could be characterized with rarefaction curves of steep slopes differing from each other (Figure 3.).

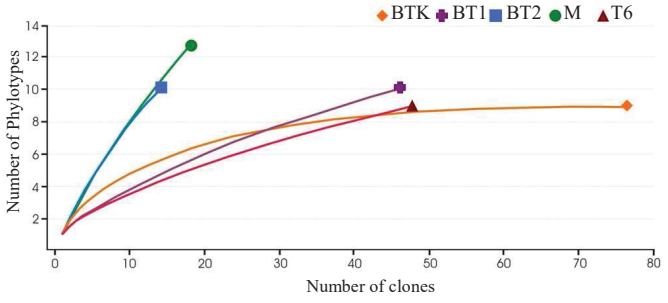

Figure 3. Rarefaction curves on the basis of the composition of clone libraries based on the 16S rDNA sequence

(BTK: composite sample taken from Büdöstapolca I and II wells; BT1: water sample taken from Büdöstapolca I well; BT2: water sample taken from Büdöstapolca II well; M: water sample taken from Matty well; T6: water sample taken from Thermal VI well)

The BTK, BT1 and T6 communities came close to the asymptote (the expected number of species) so we obtained the approximately real image about these bacterial communities, while the curves of the BT2 and M clone libraries ended sharply, so the sizes of the clone libraries were not enough large to give us an approximately real community structural image.

The community structure of the clone libraries originated from the water of the examined wells showed much similarity to many other bacteria communities originated from environments geologically far from each other but having similar geology and physical-chemical characteristics (from karstic areas, anoxic deposits, sulphurous thermal springs, sulphide water reservoirs below the ground).

It is assumed that the sulphur based bacterial metabolic processes affect and adjust the concentrations of the different dissolved sulphur forms in the well waters so they can have important role in forming the composition of the sulphurous curative waters of Harkány. Additionally, it can be supposed that intensive bio-mineralization processes take place through the metabolic processes of iron reducing and sulphate reducing bacteria during which the dissolved forms of sulphur can integrate in mineral formations as well.

The sampling and bacterial DNA extracting techniques used for our case were effective not in all cases because the biomass quantities obtained for the molecular cloning, especially for the samples from BT2 and Matty were insufficient, this fact was supported by the results of the statistical analysis of the clone libraries. By developing the efficiency of sampling larger quantity of biomass can be collected resulting clone libraries of much more clones and a detailed picture of the communities so making possible to show hidden taxa of small number.

By using *Archaea*-domain specific primers new clone libraries can be set up on the basis of 16S rRNA gene, so the phylogenetic diversity and the possible role of archaeal taxa in the chemical composition of the wells of Harkány Spa could be revealed, since it can be supposed from the presence of the lukewarm and hot water reductive sulphur and methane content as well as sulphate reducing and other thermophilic sulphur bacteria that a complex *Bacteria-Archaea* community contributes to the maintenance of the bio-geochemical cycle.

5. Publications related to the thesis

Referred scientific papers:

Miseta, R., Palatinszky, M., Makk, J., K., Borsodi, A, Márialigeti, K. (2012): Phylogenetic diversity of bacterial communities associated with sulfurous karstic well waters of a Hungarian spa. Geomicrobiology Journal, 29, 101-113. (doi: 10.1080/01490451.2011.558563) IMP: 2.017

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Miseta, R., Palatinszky, M., Márialigeti, K., Borsodi, A. (2010): A Harkányi Gyógyfürdőhöz tartozó karsztkút-vizek baktériumközösségeinek filogenetikai diverzitása. Hidrológiai Közlöny, 90 (6), 103-105.

Miseta, R., Palatinszky, M., Márialigeti, K., Borsodi, A. (2009): Molekuláris biológiai vizsgálatok a harkányfürdői kutak gyógyvizének baktériumközösségein. Hidrológiai Közlöny, 89 (6), 152-155.

Miseta, R., Palatinszky, M., Márialigeti, K., Borsodi, A. (2008): A Harkány környéki termál karsztvizek baktériumközösségei. Hidrológiai Közlöny, 88 (6), 133-137.

Othe publications:

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Miseta, R., Palatinszky, M., Márialigeti, K., Borsodi, A. (2008): Molekuláris biológiai vizsgálatok a harkányfürdői kutak gyógyvizének baktériumközösségein. (előadás; kivonat: MMT 2008. évi Nagygyűlése és XI. Fermentációs Kollokvium Absztraktfüzet p.55) Magyar Mikrobiológiai Társaság 2008. évi Nagygyűlése, Keszthely, 2008. 10. 15-17.

Miseta, R., Palatinszky., M, Márialigeti, K., Borsodi, A. (2007): Bacterial communities of thermal karstic wells of South Transdanubium. (poszter; kivonat: Acta Microbiologica et Immunologica Hungarica, 54, p.85) Magyar Mikrobiológiai Társaság XV. Nemzetközi Konferenciája, Budapest, 2007. 07. 18-20.